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# Interplay between FGF2 and BMP controls the self-renewal, dormancy and differentiation of rat neural stem cells

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## Summary

Mouse and human central nervous system progenitor cells can be propagated extensively *ex vivo* as stem cell lines. For the rat, however, *in vitro* expansion has proven to be problematic owing to proliferation arrest and differentiation. Here, we analyse the establishment, in adherent culture, of undifferentiated tripotent neural stem (NS) cell lines derived from rat foetal brain and spinal cord. Rat NS cells invariably undergo growth arrest and apparent differentiation after several passages; however, conditioned medium from proliferating cultures can overcome this block, enabling continuous propagation of undifferentiated rat NS cells. We found that dormancy is induced by autocrine production of bone morphogenetic proteins (BMPs). Accordingly, the BMP antagonist noggin can replace conditioned medium to sustain continuous self-renewal. Noggin can also induce dormant cells to re-enter the cell cycle, upon which they reacquire neurogenic potential. We further show that fibroblast growth factor 2 (FGF2) is required to suppress terminal astrocytic differentiation and maintain stem cell potency during dormancy. These findings highlight an extrinsic regulatory network, comprising BMPs, BMP antagonists and FGF2 signals, that governs the proliferation, dormancy and differentiation of rat NS cells and which can be manipulated to enable long-term clonogenic self-renewal.

**Key words:** BMP, FGF, Dormancy, Neural stem cell

## Introduction

Cultured neural stem cells with the capacity for unlimited self-renewal and functional differentiation offer an accessible model system to study neurodevelopment and neural cell biology and thus hold great interest for basic and applied neuroscience. Furthermore, advances in stem cell research are opening new perspectives on the study and treatment of neurodegenerative disease, brain injury and brain cancer. Mouse and human neural stem (NS) cell lines can be maintained long term *in vitro* without genetic modification (Reynolds and Weiss, 1992; Svendsen et al., 1998; Carpenter et al., 1999; Riaz et al., 2002; Conti et al., 2005; Pollard et al., 2006; Sun et al., 2008). These cells offer a renewable resource for neurodegenerative disease studies and are suitable for pharmaceutical and neurotoxicological screening (Conti and Cattaneo, 2010). It would be of additional value to derive NS cell lines from laboratory rats, which provide well-established models for electrophysiological, behavioural, cognitive, pharmaceutical and surgical research (Deumens et al., 2002; Jones et al., 2003; Carmichael, 2005; Kleim et al., 2007). Furthermore, transplantations into rat models provide prototypes of cell-replacement therapies for human neural disorders. Allogeneic grafts of rat neural stem cells would avoid the need for immunosuppression and ensure full compatibility of survival and trophic factors. However, despite great efforts using a wide variety of protocols, the generation of stable rat NS cell lines has proven intractable without immortalization. Rat progenitor cells derived

from various neural tissues typically sustain proliferation for only a limited period (Svendsen et al., 1997; Kelly et al., 2005). Neural progenitor cells derived from adult rat hippocampus have been expanded under the influence of fibroblast growth factor 2 (FGF2) and a glycosylated form of cystatin C (CCg), but this is associated with karyotype changes and cytogenetic abnormalities (Gage et al., 1995; Palmer et al., 1997; Taupin et al., 2000).

We have previously reported the establishment of adherent mouse and human NS cell lines from various sources (Conti et al., 2005; Pollard et al., 2006; Sun et al., 2008). Monolayer culture enables mouse and human NS cells to retain stable self-renewal and clonal tripotent differentiation capacity over extended passaging without accompanying differentiation. Here, we explore the possibility of deriving rat NS cell lines using similar culture conditions and investigate the mechanisms responsible for proliferation arrest.

## Results

### Establishment of rat foetal NS cell cultures

We followed the protocol established for foetal mouse tissue described in Conti et al. (Conti et al., 2005). Dissociated cells from E13.5 rat foetal cortex or spinal cord were plated onto laminin-coated tissue culture plastic in medium supplemented with FGF2 and epidermal growth factor (EGF). Many cells attached within 24 hours. The primary cultures were heterogeneous; they contained nestin-positive precursor cells, TuJ1-positive neurons and a small

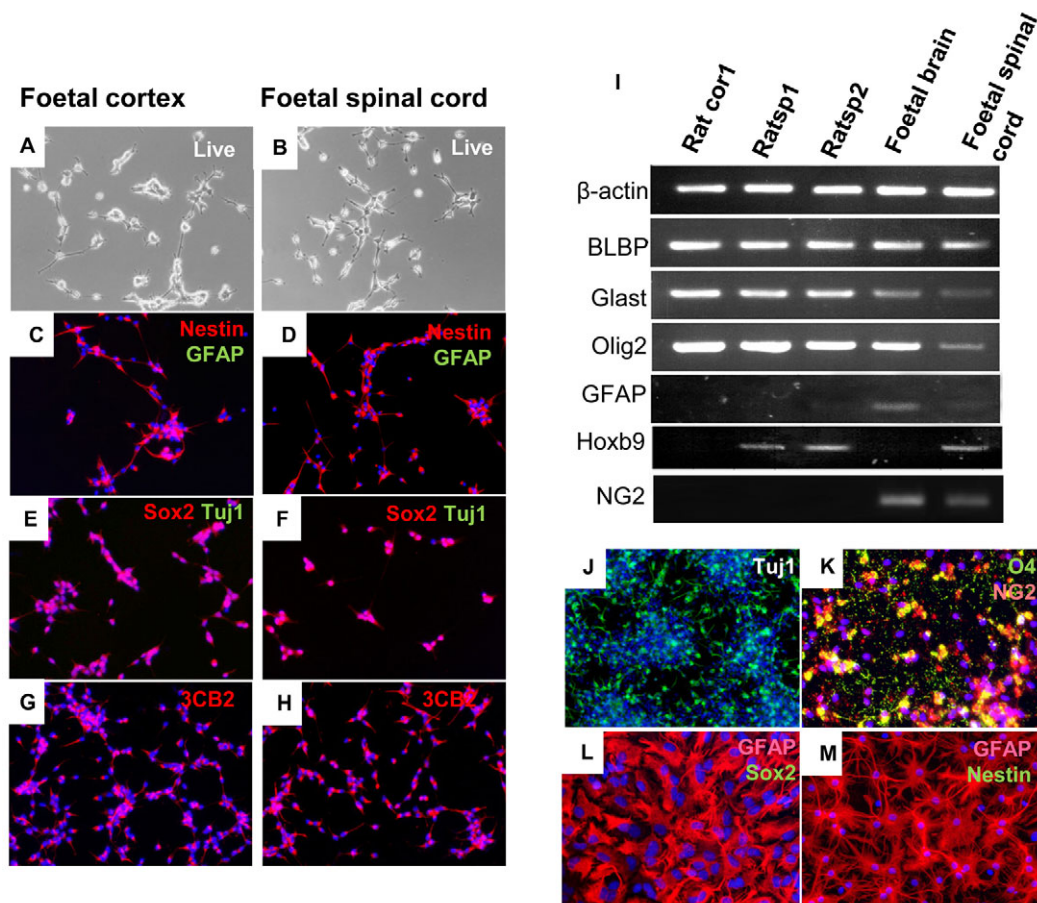
number of GFAP-expressing astroglial cells (data not shown), as previously reported for initial stages of mouse and human NS cell derivation (Conti et al., 2005; Sun et al., 2008). To enrich the undifferentiated cells, we transferred primary cultures onto gelatin-coated dishes on day 5 after initial plating. Differentiated neurons failed to survive after passaging and cultures progressively became dominated by proliferating cells (Fig. 1A,B). By 3 weeks, the cultures were homogeneously nestin-positive and negative for the differentiation markers TuJ1 and GFAP. At this point they were considered to be rat NS cells at passage 1 (Fig. 1C–F). Using this protocol, from independent foetal cortices, we derived three rat NS cell cultures (Ratcor1, Ratcor2 and Ratcor3) and, in addition, two cultures from foetal spinal cords (Ratasp1 and Ratasp2). We expanded these cultures by renewing culture medium every 2 days and splitting cells at a ratio of 1:3 once they became confluent.

Rat NS cells are morphologically similar to their mouse counterparts. In the presence of EGF and FGF2, they exhibit bipolar morphology and are motile. Immunostaining and RT-PCR indicated that rat NS cells expressed a set of neural progenitor and/or radial glia markers, including nestin, Sox2, 3CB2, BLBP, Glast, Olig2 and vimentin, similar to mouse and human cells (Fig. 1C–I) (Conti et al., 2005; Pollard et al., 2006; Sun et al., 2008). Differentiation markers for neurons (TuJ1) and astrocytes (GFAP), were not detected

under expansion conditions (Fig. 1C–F). The oligodendrocyte precursor cell (OPC) marker NG2 was also absent from propagating NS cells (Fig. 1I; data not shown). Spinal-cord-derived cells expressed the posterior marker Hoxb9 but were otherwise similar to cortex-derived cells. When cultured under differentiating conditions (Conti et al., 2005; Glaser et al., 2007), rat NS cells at passages 4–10 were able to generate neurons (TuJ1-positive), oligodendroglia (O4- and NG2-positive) and astrocytes (GFAP-positive) (Fig. 1J–M). In three independent experiments, a total of 6340 cells were scored to determine the neuronal and oligodendroglial differentiation efficiencies, respectively. In total, 38.6(±4.3)% TuJ1-positive cells were obtained with the neuronal protocol, whereas 19.6(±3.2)% O4-positive cells were generated with the oligodendroglial protocol. Most strikingly, upon withdrawal of EGF and FGF2 and exposure to BMP4 (10 ng/ml) or serum (3%), rat NS cells consistently produced a uniform population of GFAP-positive astrocytes with spread morphology. Neural precursor markers, such as nestin and Sox2, were absent in these astrocytes (Fig. 1L,M).

### Conditioned medium enables continuous expansion of rat NS cells

We next investigated whether rat NS cells could be expanded as stable cell lines. Unlike mouse and human NS cells, which undergo



**Fig. 1. Derivation of rat foetal NS cells.** Rat NS cells can be derived from both foetal cortex (A) and spinal cord tissue (B). In expansion cultures, rat NS cells express nestin, Sox2 and 3CB2, but they do not express GFAP or TuJ1 (C–H). RT-PCR indicates that rat NS cells also express mRNA encoding BLBP, Glast and Olig2, but not NG2. Spinal cord NS cells express mRNA encoding Hoxb9, which is not detected in cortical NS cells (I). Rat NS cells are able to generate neurons (TuJ1-positive), oligodendroglia (O4-positive and NG2-positive) and astrocytes (GFAP-positive and Sox2-negative) when cultured under the respective differentiation conditions (J–M).

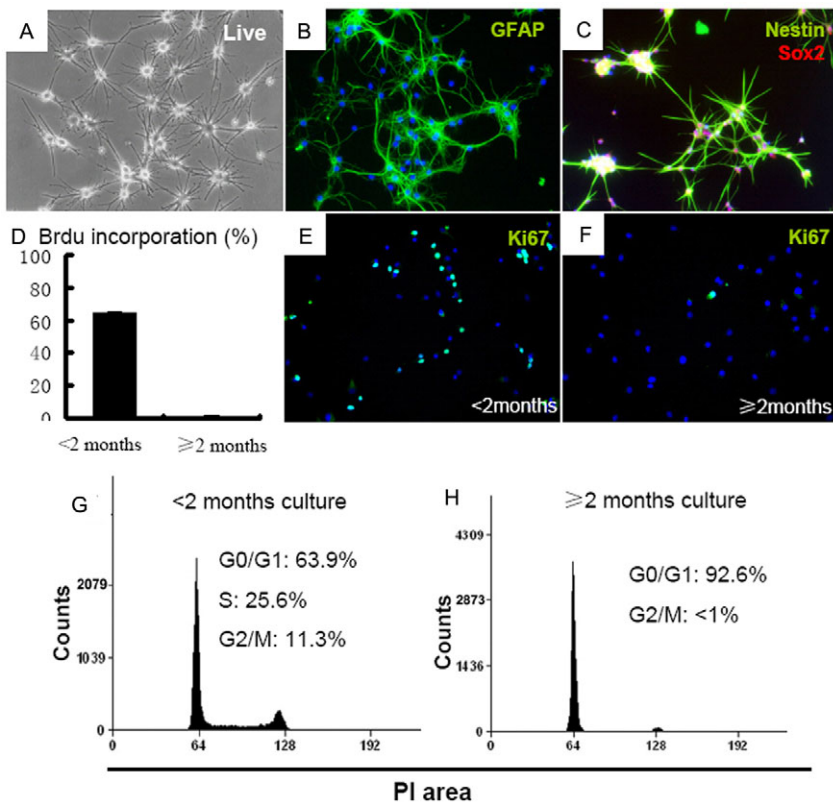
continuous propagation in the presence of EGF and FGF2, we found that rat NS cells spontaneously ceased cell proliferation after ~30 generations, or around passage 10. This was observed with the same timing in all of the five cultures that had been independently established and maintained. Coincident with proliferation arrest, cells developed a stellate morphology and showed upregulation of the astroglial marker GFAP. However, in contrast to the astrocytes generated upon growth factor withdrawal, these cells retained expression of the neural precursor markers nestin and Sox2 (Fig. 2A–C). Furthermore, they did not express the astroglial marker S100 $\beta$ . Immunostaining showed that very few of the cells expressed Ki67 and <1% of cells were labelled with BrdU after 24 hours of incubation. By contrast, in cultures at earlier passages, 45.3( $\pm$ 2.4)% of the cells expressed Ki67 and 64.4( $\pm$ 1.9)% of cells incorporated BrdU after 24 hours (Fig. 2D–F). Propidium iodide (PI) staining and flow cytometry analysis revealed that >90% of stellate cells were in the G0 or G1 phase of cell cycle (Fig. 2G,H). These stellate cells, expressing GFAP, nestin and Sox2, were viable for at least 2 months. Crucially, we found that the stellate phenotype and sustained expression of nestin and Sox2 depends upon EGF and FGF2 exposure. When EGF and FGF2 were withdrawn from the culture medium, many cells died and those surviving differentiated into flat spread astrocytes that were GFAP-positive but negative for nestin and Sox2 (data not shown).

Conditioned medium has been reported to maintain the propagation of adult rat hippocampal progenitor cells (Gage et al., 1995; Palmer et al., 1997). We therefore tested whether maintaining rat NS cells at higher densities, with a 50% medium exchange every 3 days, would bypass the proliferation arrest by self-conditioning. Indeed, we found that the generation of stellate cells was suppressed and NS cells exhibited stable proliferation beyond

10 passages. These cells maintained homogeneous nestin, Sox2, BLBP, vimentin and Olig2 expression (Fig. 3A). Tuj1 and GFAP expression were fully suppressed, and cultures continued to proliferate with no change in doubling time over multiple passages. Notably, and regardless of passage number, if conditioned medium was withdrawn, rat NS cells progressively ceased division and developed stellate morphology within 2 weeks.

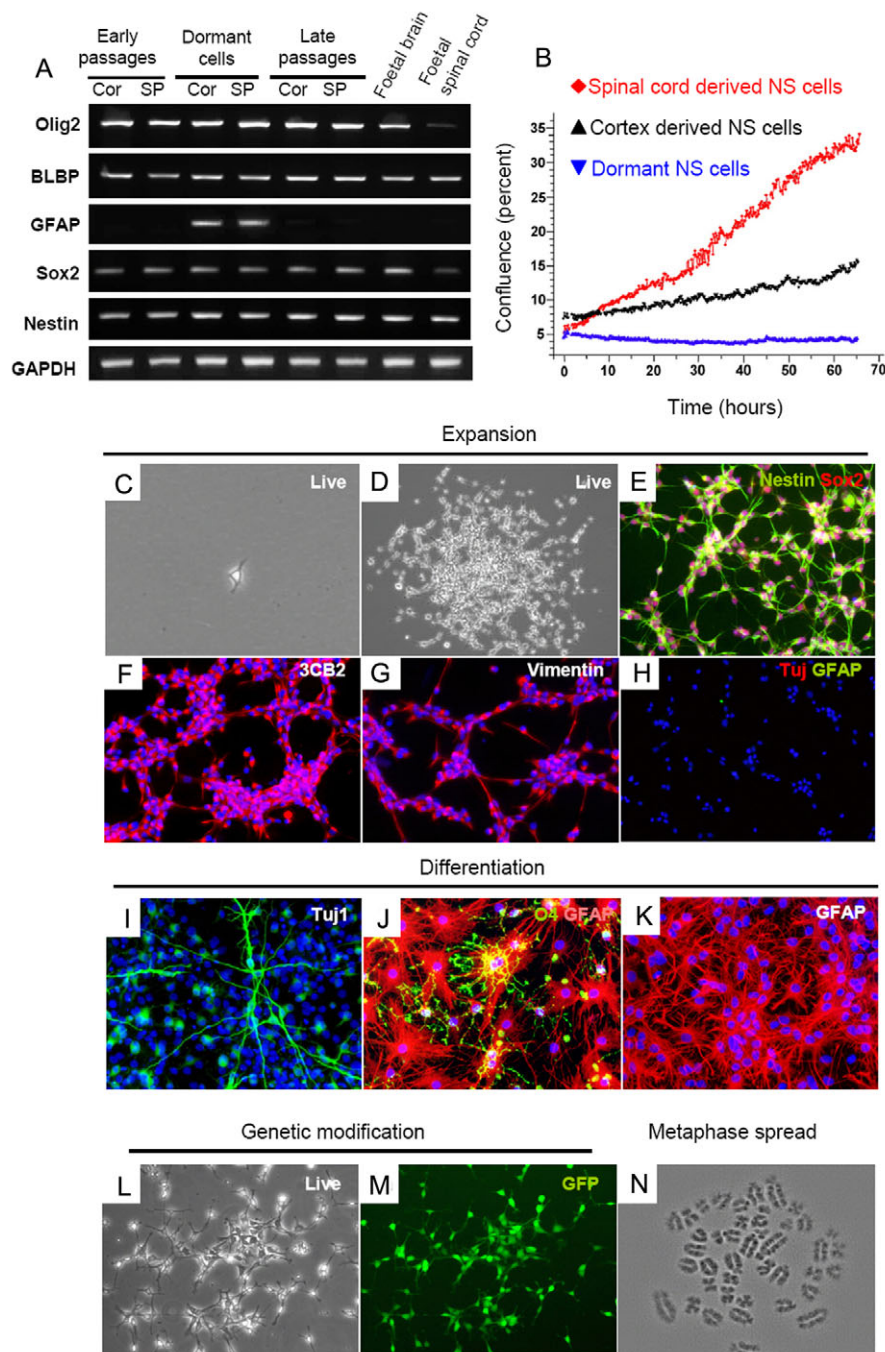
Although they were morphologically indistinguishable, rat cortical NS cells divided slower (doubling time of 48–72 hours) than spinal cord cells (doubling time of ~24 hours) (Fig. 3B). To date, using these self-conditioned medium protocols, we have expanded rat foetal-cortex- and spinal-cord-derived NS cells for more than 25 passages ( $\geq$ 75 generations) or 50 passages ( $>$ 150 generations), respectively. The rat NS cells expanded in conditioned medium were clonogenic; when single rat NS cells were seeded into 96-well plates, 8.33% (16 of 192) generated colonies in conditioned medium (Fig. 3C,D), whereas control plates using non-conditioned medium only led to small dormant colonies. We picked three colonies in the conditioned medium at random and found each of them could be further expanded into clonal cell lines. The clonal cell populations were indistinguishable from their parental cell lines in terms of culture requirements, marker expression and tripotent differentiation potential (Fig. 3E–K).

Upon treatment with conditioned medium, we found that established rat NS cell lines could be genetically modified. For example, when Ratsp2 NS cells were transfected with linearized pCAG-GFP-IP, by nucleofection, ~30% of cells displayed green fluorescent protein (GFP) expression 30 hours later. Cells with stable and readily visualized GFP were selected and further expanded as a Ratsp2-GFP NS cell line (Fig. 3L,M). Because previous long-term expansion of rat neural progenitors has been accompanied by karyotypic changes, we examined the



**Fig. 2. Rat NS cells enter dormancy.** Rat NS cells spontaneously entered dormancy after 8 weeks expansion. NS cells at passage 10 displayed stellate morphology and GFAP expression (A,B). However, they retained neural precursor marker expression, such as for nestin and Sox2 (C). Unlike proliferative NS cells at earlier passages, BrdU incorporation and Ki67 staining indicated that the stellate cells did not usually divide (D–F), which was supported by PI staining and flow cytometry analysis, showing the majority of cells were in G0 or G1 phase (G,H).





**Fig. 3. Continuous self-renewal of rat NS cells depends upon conditioned medium.** When cultured in conditioned medium, rat NS cells remain proliferative and do not enter dormancy. Rat cortex (Cor) and spinal cord (SP) NS cells at late passages exhibit similar gene expression to early passage cells (A), although cortical NS cells proliferate slower than spinal cord cells (B). In the presence of conditioned medium, clonal cell lines that exhibited continuous self-renewal (C–H) and trilineage differentiation according to culture conditions (I–K) could be established from single rat NS cells. Fluorescent reporter lines were established by stable transfection of the pPyCAGGFPIIP plasmid (L,M). Metaphase spreads show rat spinal cord NS cells at passage 45 have 42 chromosomes (N).

chromosome complement of expanded NS cells. From analysis of 173 metaphase spreads, ~82% of cortical cells at passage 25 and 85% of spinal cord cells at passage 45 had the expected 42 chromosomes (Fig. 3N); ~15% of cells displayed fewer than 42 chromosomes but this might partly be because not all chromosomes were released from the nucleus or that individual chromosomes were not all resolved.

We then tested the effect of conditioned medium on spontaneously arrested stellate cells. We transferred cultures that had been non-proliferative for over 4 weeks (confirmed by parallel Ki67 staining), into conditioned medium from proliferative cultures supplemented with fresh EGF and FGF. We observed that the non-proliferating stellate cells resumed proliferation, regained bipolar

morphology and rapidly re-established undifferentiated cell populations (supplementary material Movie 1). Cultures recovered from arrested cells exhibited the same marker expression and potential for self-renewal and differentiation as NS cells prior to arrest (data not shown). This response was specific to the GFAP-positive stellate cells; astrocytes that were GFAP-positive and nestin- and Sox2-negative, generated by FGF and EGF withdrawal and exposure to serum or BMP, did not re-enter the cell cycle or change morphology in conditioned medium. These results establish that GFAP-positive stellate cells, in the presence of FGF2 and EGF, are functionally distinct from GFAP-expressing cells induced by serum or BMP in the absence of growth factors, which are probably terminally differentiated.

### Cystatin C, Wnt and PDGF signals do not overcome rat NS cell dormancy

The observation that conditioned medium overcomes spontaneous cell dormancy indicates that this is not a cell intrinsic phenomenon but that it is governed by microenvironmental factors. We reasoned that there should be autocrine or paracrine factor(s) regulating NS cell proliferation. CCg has been reported to be an FGF cofactor that promotes proliferation of adult rat hippocampal progenitor cells (Taupin et al., 2000). We found that cystatin-C-encoding mRNA was present in both proliferative and dormant rat NS cells (supplementary material Fig. S1A). However, addition of CCg, at either 10 nM or 20 nM (final concentration), into fresh culture medium could not replace conditioned medium to sustain proliferation of rat NS cells. When we replaced conditioned medium with fresh expansion medium supplemented with CCg (10 nM or 20 nM final concentration), proliferative rat NS cells arrested and became stellate within 2 weeks (data not shown). Wnt proteins have previously been recognized as growth factors that in vivo promote central nervous system (CNS) progenitor proliferation and enhance neurogenesis (Chenn and Walsh, 2003; Willert et al., 2003; Zechner et al., 2003; Hirabayashi et al., 2004; Reya and Clevers, 2005). In our cultures, we found rat NS cells expressed mRNAs encoding Wnt4 and Wnt5a, as well as Wnt receptors, including frizzled 1 (Fzd1), Fzd4 and Fzd9 (supplementary material Fig. S1A). However, immunostaining indicated that  $\beta$ -catenin, a key component in the canonical Wnt signalling pathway, was not localized to the nucleus in either proliferative or dormant NS cells (supplementary material Fig. S1B). We treated rat NS cells with TWS119, a glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) inhibitor (Ding et al., 2003), to stimulate nuclear translocation of  $\beta$ -catenin (supplementary material Fig. S1C), but this treatment did not suppress spontaneous cell dormancy in fresh expansion medium. In addition, TuJ1-positive cells appeared after TWS119 exposure, suggesting stimulation of neuronal differentiation (supplementary material Fig. S1C). We also applied Wnt3a (10 ng/ml) and/or Wnt5a (80 ng/ml) in fresh expansion medium, but cells still became dormant within 2 weeks after the withdrawal of conditioned medium (data not shown). Finally, we found that addition of the Wnt receptor antagonists dickkopf-related protein 1 (Dkk1; 0.2  $\mu$ g/ml) or secreted frizzled related protein 4 (sFRP4; 10  $\mu$ g/ml) in conditioned medium did not reduce the proliferation of rat NS cells over a 2-week period (data not shown).

Previous studies have shown that, in addition to its recognized roles promoting survival and proliferation of oligodendroglia, platelet-derived growth factor (PDGF) signalling might regulate the fate choice between glial and neuronal lineages in adult neural precursor cells (Johe et al., 1996; Williams et al., 1997; Jackson et al., 2006). We found that rat NS cells expressed mRNAs encoding PDGF and the  $\alpha$ -type PDGF receptor (PDGFR $\alpha$ ) that these were upregulated in dormant cells (supplementary material Fig. S1A). However, applying recombinant PDGF or the PDGFR inhibitor AG1295/96 (Banai et al., 1998; Tse et al., 2002) did not affect rat NS cell proliferation in conditioned medium nor inhibit the generation of dormant cells in fresh medium. Collectively, these observations indicate that CCg, Wnt and PDGF signalling are not limiting for continuous expansion of rat NS cells.

### BMP antagonists sustain long-term proliferation of rat NS cells

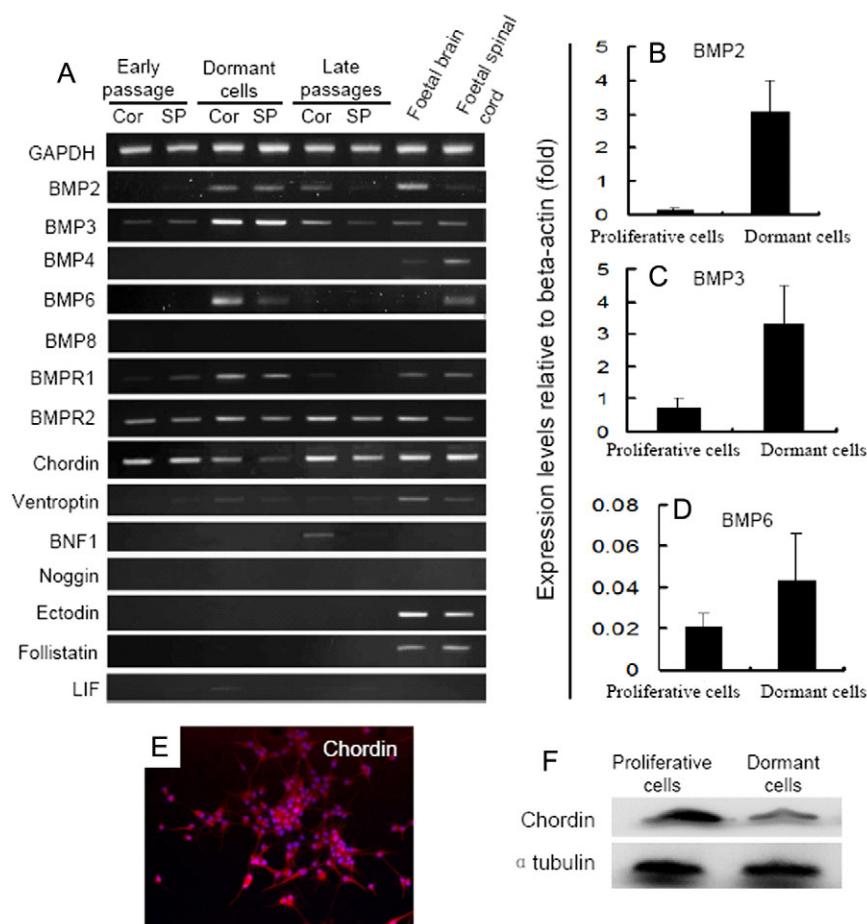
On the basis of the fact that dormant rat NS cells express GFAP, a marker for astroglia cells, we evaluated whether accumulation of

GFAP-inducing factors, such as LIF and BMPs (Gross et al., 1996; Nakashima et al., 1999; Bonaguidi et al., 2005; Weible and Chan-Ling, 2007), triggered the transition to dormancy. Indeed, although we did not detect LIF expression, RT-PCR indicated that rat NS cells expressed BMP2, BMP3, BMP6 and BMP receptors, and that their expression is, in general, upregulated in dormant compared with proliferating cells (Fig. 4A–D). In addition, we found that rat NS cells also expressed the BMP antagonist chordin, verified by RT-PCR, immunostaining and western blots (Fig. 4A,E,F). Interestingly, western blots indicated that chordin expression was downregulated in dormant cells (Fig. 4E,F). We therefore speculated that rat NS cell cultures are ‘poised’ between positive and negative regulators of the BMP signalling pathway.

To investigate whether BMP signals are involved in NS cell dormancy, we plated proliferative cells in expansion medium and supplemented this with 10 ng/ml BMP4. To minimize the effects of autocrine factors, cells were plated at a lower density ( $\sim 2 \times 10^3$  cells per cm<sup>2</sup>) and culture medium was renewed by 100% daily. In parallel, control cells were plated at the same density in conditioned medium. In the presence of BMP, cells rapidly ceased division and developed a stellate morphology within 3 days (Fig. 5A). In the parallel conditioned medium cultures, cells remained proliferative and had a undifferentiated morphology. Immunostaining showed that rat cells exposed to BMP4 in expansion medium coexpressed GFAP with nestin and Sox2 (Fig. 5B,C). PI staining and flow cytometry confirmed that  $\sim 90\%$  of the BMP4-induced stellate cells were in G1 or G0 phase (Fig. 5D), similar to spontaneously arising dormant cells. We found that BMP4 could induce cell dormancy at any culture stage, including at early passages prior to spontaneous cell cycle arrest. Most importantly, when BMP4 was withdrawn for 48 hours and cultures were supplemented with conditioned medium from proliferative cultures, the BMP-induced stellate cells re-entered the cell cycle and expanded as bipolar undifferentiated NS cells. RT-PCR indicated that rat NS cells also expressed other TGF $\beta$  superfamily members such as TGF $\beta$ 1 and 2, activin A and B, GDF11 and 15, and their corresponding receptors (supplementary material Fig. S2A). However, addition of TGF $\beta$ 1, activin B and GDF11 proteins into expansion medium, either individually or in combination, did not lead to cell cycle arrest or upregulation of GFAP (supplementary material Fig. 2B–J).

We next tested whether conditioned medium from proliferative rat NS cells had BMP antagonist activity. When rat NS cells were cultured in conditioned medium supplemented with BMP4 at up to 100 ng/ml (ten times higher than used to induce dormancy), only a small fraction ( $\sim 4\%$ ) of cells became GFAP positive (Fig. 5E,F). The majority of cells remained proliferative and nestin- and Sox2-positive (Fig. 5G). Furthermore, rat NS cells could form undifferentiated colonies when plated at low density in BMP4 in conditioned medium. By contrast, no colonies formed in fresh medium in the presence of BMP4.

We investigated whether a combination of BMP antagonists and fresh expansion medium could replace conditioned medium to propagate rat NS cells. Because recombinant chordin does not have consistent biological activity, we applied an alternative BMP antagonist, noggin (Smith and Harland, 1992). In the presence of noggin, we found rat NS cells could bypass spontaneous dormancy without recourse to conditioned medium. In addition, we found that fresh expansion medium containing noggin could induce dormant stellate cells to re-enter the cell cycle. Rat NS cells cultured with noggin retained stable proliferation for at least 25 passages ( $>75$  generations) and expressed nestin and Sox2



**Fig. 4. Dormant NS cells express BMPs and chordin.** RT-PCR profiling indicates that dormant rat NS cells from the cortex (Cor) and spinal cord (SP) express mRNA encoding BMP2, BMP3, and BMP6 along with BMP receptors (A). qRT-PCR confirms upregulation of BMP transcripts in quiescent cells (means $\pm$ s.d.,  $n=5$ ) (B–D). Rat NS cells also express the BMP antagonist chordin, as detected by RT-PCR (A), immunostaining (E) and immunoblotting (F).

uniformly with no expression of GFAP (Fig. 5H–J). These cultures remained competent for differentiation. In particular, clonal lines established in noggin were able to generate neurons, astrocytes and oligodendrocytes (Fig. 5K–M). Thus, adding noggin to rat NS cell culture medium is an effective means to maintain their long-term expansion and potency.

#### BMPs induce rat NS cell dormancy through Smads

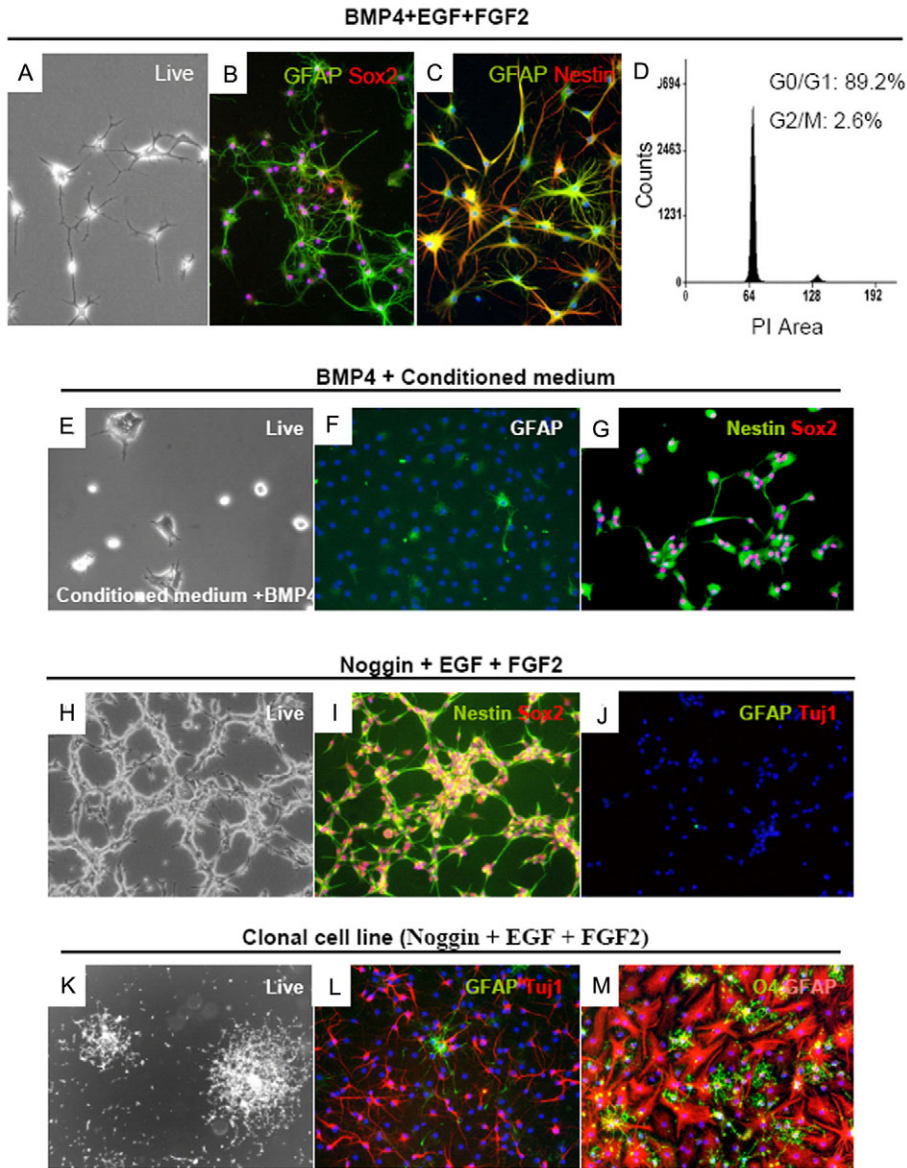
To investigate further the downstream mechanisms of how BMP signals induce rat NS cell dormancy, we analysed the two well-described BMP signalling pathways: the ‘canonical’ BMP-Smad cascade, in which BMP signals are mediated by R-Smads (Smad1, 5 and 8) and co-Smad (Smad4), and the ‘non-canonical’ BMP-MAPK cascade in which BMP signals are transduced by TAB1 and TAK1, which activate JNK and p38 MAPK signalling (Yamaguchi et al., 1995; Shibuya et al., 1998; Kimura et al., 2000; von Bubnoff and Cho, 2001). Immunostaining and western blots indicated that proliferative rat NS cells displayed negligible phosphorylation of Smad1, 5 and 8 (Fig. 6A,D). By contrast, both spontaneous and BMP4-induced dormant NS cells displayed readily detectable phosphorylated R-Smads, along with a slight increase in total Smad levels, which was also seen at the mRNA level by quantitative real-time PCR (qRT-PCR) (Fig. 6B–E). p38 phosphorylation was also observed in dormant cells (Fig. 6F). However, addition of the p38 inhibitors SB203580 (Saklatvala et al., 1996) or PD169316 (Gallagher et al., 1997) did not prevent NS cells entering dormancy (Fig. 6G). These observations suggest

that BMPs induce NS cell dormancy through the BMP-Smad cascade.

#### FGF2, but not EGF, maintains the stem cell potency of dormant NS cells

BMPs induce terminal astrocyte differentiation in the absence of EGF and FGF2. We investigated whether both the EGF and FGF2 growth factors are necessary to maintain stem cell potency. Rat NS cells cultured in fresh medium supplemented with BMP4 together with EGF only, rapidly became non-proliferative, nestin- and Sox2-negative and GFAP-positive (Fig. 7A–C). After being plated back into conditioned medium or expansion medium together with noggin, these nestin- and Sox2-negative and GFAP-positive cells did not re-enter the cell cycle nor express Nestin or Sox2, indicating they had lost their stem cell character and had differentiated into astrocytes. By contrast, in cultures supplemented with BMP together with FGF2 only, cells ceased division and developed GFAP expression, but with the majority retaining nestin and Sox2 expression (Fig. 7D–E). When BMP4 was withdrawn, these Nestin-, Sox2- and GFAP-positive cells could re-enter the cell cycle and establish an undifferentiated NS cell population. In addition, when FGF2- and BMP4-induced dormant cells were directly plated into appropriate differentiation conditions, they could generate astrocyte, neurons and oligodendrocytes (Fig. 7G–I). Therefore, we conclude that exogenous FGF2 plays the central role in suppressing terminal astroglial differentiation and maintaining stem cell potency in the presence of BMP.





**Fig. 5. Continuous self-renewal of rat NS cells requires antagonism of BMP.** In the presence of EGF and FGF2, exogenous BMP4 inhibited the proliferation of rat NS cells and induced rat NS cells to develop stellate morphology (A,D). The BMP4-induced stellate cells express GFAP, nestin and Sox2 (B,C). In the presence of conditioned medium, exogenous BMP4 could not induce NS cell dormancy. Rat NS cells exposed to BMP4 at 100 ng/ml still proliferated and generated colonies when plated at low density (E). Immunostaining show that ~4% of cells expressed GFAP under this condition (F), and all cells retained nestin and Sox2 expression (G). Addition of the BMP antagonist noggin and fresh expansion medium allow continuous propagation of rat NS cells. Ratsp1 NS cells at passage 44 exhibit nestin and Sox2 expression and are GFAP- and Tuj1-negative (H–J). Clonal NS cells expanded in the presence of noggin are tripotent, as determined by transfer to the respective differentiation cultures (K–M).

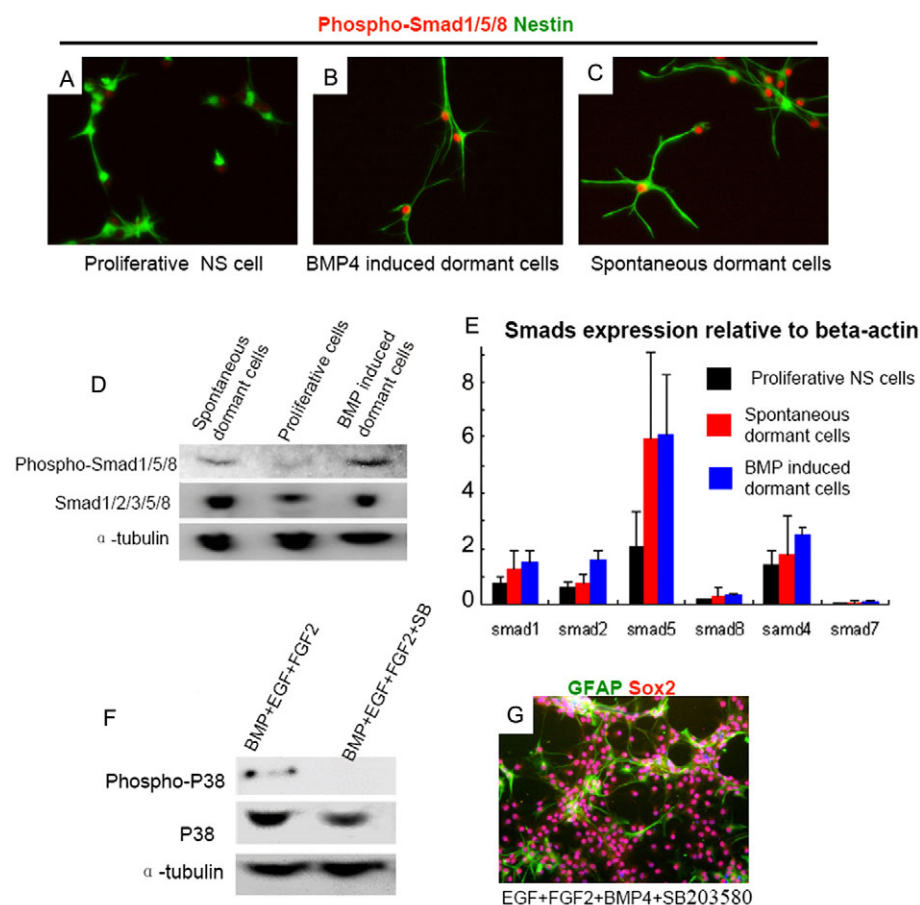
## Discussion

Here, we have described the long-term expansion of tripotent and clonogenic rat foetal NS cell lines by overcoming autocrine BMP stimulation. Our results indicate that BMP induces NS cell growth arrest through Smads, but, in the presence of FGF2, terminal differentiation is blocked and stem cell potency preserved. These findings indicate that NS cell propagation, dormancy and differentiation are regulated by counterbalancing BMP and FGF signals. In foetal rat NS cell cultures, we consistently observed that spontaneous cell dormancy occurs ~8 weeks (~passage 10) after initial plating. This timing could reflect operation of an intrinsic clock in the neural progenitors, such that their properties alter in a temporally determined manner (Panchision et al., 2001). However, the observation that exogenous BMP4 can induce dormancy precociously demonstrates that cells are competent at all stages to respond to this signal. Primary NS cell cultures are not homogenous, and as the cell population evolves the balance of BMPs and BMP antagonists might change. It also remains possible that NS cells alter their responsiveness to BMP levels over time. The timing of

spontaneous arrest might therefore arise from a combination of an altered extrinsic balance combined with an increased intrinsic sensitivity to BMPs. Crucially, however, there is no cell autonomous loss of self-renewal or potency.

Interestingly, EGF and FGF2 are not equivalent in their effects on rat NS cells. EGF is the key mitogen for self-renewal of mouse and human NS cells (Pollard et al., 2006; Sun et al., 2008), but it is FGF2 that exhibits the major ‘anti-BMP’ effect and sustains stem cell potency in dormant rat NS cells. Cross-regulation by FGFs and BMPs has previously been described in the CNS and other areas of the body, such as tooth and limb (Niswander and Martin, 1993; Neubuser et al., 1997; Lillien and Raphael, 2000). Possible mechanisms for the modulatory effects of FGFs on BMP signalling are phosphorylation and inhibition of Smads by MAPKs (Kretzschmar et al., 1997; von Bubnoff and Cho, 2001; Pera et al., 2003). However, although pharmacological blockade of the FGFR resulted in terminal astrocyte differentiation in the presence of BMP, cells remained nestin-positive when only activation of ERK1/2 was blocked using the selective inhibitor PD0325901





**Fig. 6. BMPs induce rat NS cell dormancy through Smads.** Proliferating rat NS cells show barely detectable levels of phosphorylated Smad1, 5 and 8 but this is evident in almost all nuclei of dormant cells (A–D). qRT-PCR (means±s.d.,  $n=5$ ) (E) and immunoblotting (D) show that the expression level of effector Smads also slightly increases in dormant cells. Although BMP signals also induce phosphorylation of the p38 MAPK, inhibition of p38 by SB203580 does not prevent BMP-induced cell dormancy (F,G).

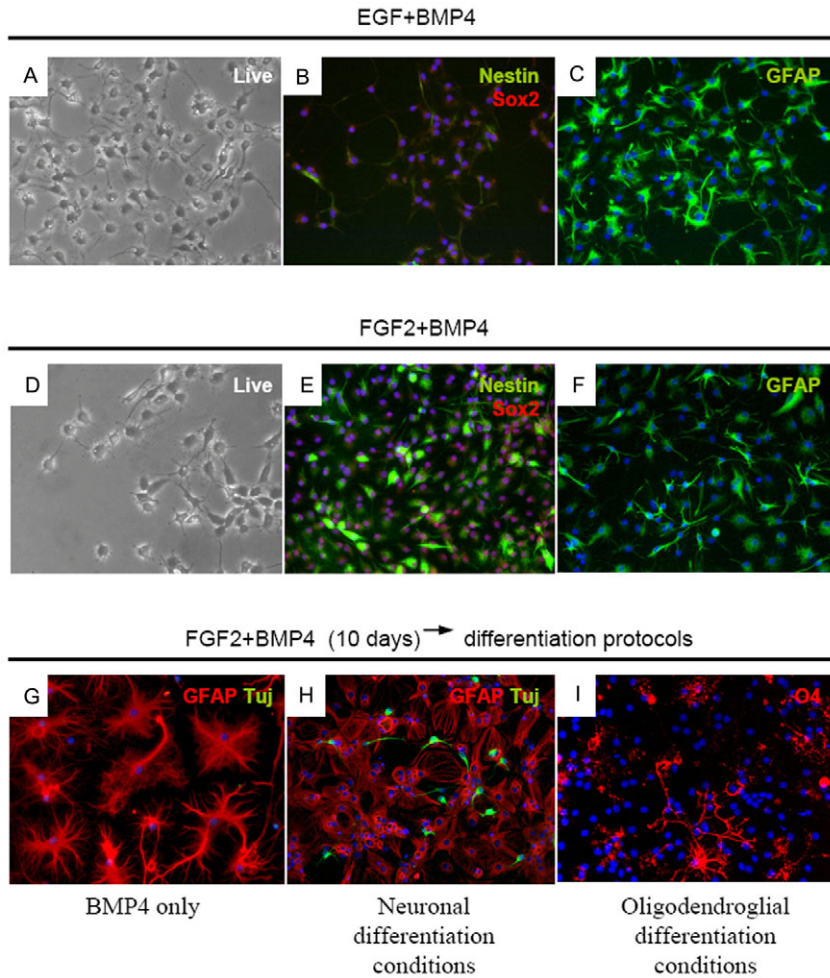
(data not shown). This suggests that FGF stimulation of phosphoinositide 3-kinase signalling might be a crucial determinant of dormancy, probably acting through Akt (Sinor and Lillien, 2004).

We observed distinctive behaviour during efforts to extend these observations to derivation of adult rat NS cells from the subventricular zone (SVZ) and dentate gyrus (DG). In medium supplemented with noggin, adult neural progenitor cells remained proliferative for at least 2 months without developing GFAP expression (supplementary material Fig. S3). However, unlike foetal NS cells these cultures were overtly heterogeneous with ~20% of cells spontaneously differentiating into O4-positive cells (supplementary material Fig. S3F). This appeared to be due to autocrine and/or paracrine PDGF stimulation because addition of the PDGFR inhibitors AG1295 or AG1296 reduced oligodendrocyte generation. However, these chemicals also led to significant cell detachment, and therefore the monolayer cultures could not be maintained. It is possible that more selective inhibitors or genetic perturbation of the PDGF pathway would enable robust propagation of adult rat NS cells.

BMP signalling activates NFATc1 (nuclear factor of activated T cells c1) and represses cyclin-dependent kinase 4 (CDK4) expression to maintain stem cell quiescence in the hair follicle (Horsley et al., 2008). However, in rat NS cells we found that expression of *CDK2*, *CDK4* and *CDK6* mRNAs were either unchanged or only slightly upregulated when NS cells entered dormancy (supplementary material Fig. S4A). We also investigated *Id* genes, well-described BMP targets in various cell types including

neural progenitors (Benezra et al., 1990; Hollnagel et al., 1999; Nakashima et al., 2001; Lopez-Rovira et al., 2002; Ying et al., 2003). qRT-PCR indicated that *Id1*, *Id2* and *Id3* were barely detectable in proliferating rat NS cells and that their expression does not change significantly when NS cells enter dormancy (supplementary material Fig. S4A). qRT-PCR also indicated that the expression of *EGFR* and *FGFR1* actually increased in dormant cells (supplementary material Fig. S4A). We also examined Akt and PTEN. Western blots showed the protein expression and phosphorylation of these factors exhibited little change between proliferating rat NS cells and dormant cells (supplementary material Fig. S4B). Finally, we examined CDK inhibitors. Although expression of *p16* (*Cdkn2a*), *p19* (*Cdkn2d*) and *p27* (*Cdkn1b*) were either at a negligible level or unchanged in dormant compared with proliferative cells, we found that *p21* (*Cdkn1a*) was upregulated over tenfold in dormant NS cells (supplementary material Fig. S4A). Therefore p21 might play a major role downstream of BMP in NS cell growth arrest.

Dormant NS cells express GFAP and show morphological features of differentiation. However, they retain neural precursor markers and can readily resume self-renewal. We speculate that dormant NS cells represent a transitional state between NS cells and astrocytes. BMPs might trigger the first step(s) of astroglial differentiation, resulting in cell cycle arrest and GFAP expression, while the generation of more mature astrocytes is inhibited by the action of FGF2, which sustains stem cell potency. This presents a note of caution for the concept that BMP or BMP mimetics might be sufficient as a differentiation therapy targeting brain tumour



**Fig. 7. FGF2 sustains the stem cell character of dormant NS cells in the presence of BMP.** In cultures supplemented with EGF and BMP4 without FGF2, NS cells shown downregulation of nestin and Sox2 expression and differentiated into GFAP-positive astrocytes, rather than entering dormancy (A–C). By contrast, although NS cells also developed GFAP expression in cultures supplemented with FGF2 and BMP4, almost all cells retained nestin and Sox2 expression (D–F). When the FGF2- and BMP4-induced dormant cells were directly plated into astrocyte, neuronal or oligodendroglial differentiation conditions, they could generate uniform GFAP-positive and nestin- and Sox2-negative astrocytes (G), ~10–15% Tuj1-positive neuronal cells (H), or a similar proportion of O4-positive oligodendrocytes (I), respectively.

stem cells (Piccirillo et al., 2006). Although it is well-established that BMPs can promote astroglial differentiation (Mabie et al., 1999; Nakashima et al., 1999; Lim et al., 2000; Mehler et al., 2000; Hebert et al., 2002), by analysing conditional deletion of Smad4 or infusion of noggin in the adult mouse subependymal zone, Colak and colleagues (Colak et al., 2008) concluded that BMP signalling is also required for adult neurogenesis. It has also been shown that noggin can expand hippocampal progenitors in the subgranular zone (Bonaguidi et al., 2008). While our study was under review, Mira and colleagues presented evidence that BMP induces quiescence in adult hippocampal cells in the subgranular zone (Mira et al., 2010). Furthermore, and in line with our present observations, they also noted active Smad signalling and upregulation of p21 in quiescent neural stem cells. Collectively these findings indicate that neurogenic niches in the rodent brain (Doetsch et al., 1999; Alvarez-Buylla et al., 2001; Seri et al., 2001) are maintained by BMP regulation of the quiescent stem cell population.

In conclusion, our findings reveal that BMP signalling plays a central role in determining rat NS cell dormancy. By antagonizing BMP signals, long-term propagation of rat NS cells can be achieved, providing a new resource for both basic and applied research. Furthermore, rat NS cell dormancy might constitute an *in vitro* analogue of neural stem cell quiescence in adult brain and thus provide a tractable experimental system for interrogating the molecular regulation of stem cell homeostasis and activation.

## Materials and Methods

### Cell culture

Fischer 344 and Sprague Dawley rats were employed in this study. Animals were maintained and used in a designated facility under licences issued by the UK Home Office. We obtained fresh neural tissue from rat embryos at embryonic day 13.5 or ~3-month-old adult rats using dissection protocols described elsewhere (Conti et al., 2005; Pollard et al., 2006). Foetal and adult tissue samples were incubated with a mix of PBS and Accutase (1:1) at 37°C for 5 minutes and mechanically dissociated. Cell suspensions were then plated onto laminin- or gelatin-coated dishes in expansion medium comprising Euromed-N (Euroclone) or RHB-basal medium (Stem Cell Sciences), L-glutamine (2 mM final; Gibco), modified N2 supplement (Ying and Smith, 2003), penicillin-streptomycin (10 ml/l final; Sigma) and 10 ng/ml of both mouse EGF (Peprotech) and human FGF2 (Peprotech). To obtain stable foetal rat NS cell lines, cultures were maintained at relatively high densities (50–80% confluence) in self-conditioned medium obtained by renewing 50% of the culture medium every three days. In order to culture rat NS cells without self-conditioning, we collected expansion medium that had been exposed to proliferative rat NS cells for at least 3 days. After filtering this through 40 µm cell strainers (BD), conditioned medium was diluted with an equal volume of fresh medium, supplemented with growth factors and supplements at the final concentrations listed above and applied to cells. Incubation of empty plates in conditioned medium confirmed that there was no carryover of cells. Alternatively, rat NS cells were expanded in fresh expansion medium supplemented with noggin (50 ng/ml; Peprotech). Higher noggin concentrations are required when cells are plated at low density, particularly for brain-derived NS cells.

Differentiation and clonal assays were performed following protocols for mouse NS cells (Conti et al., 2005; Glaser et al., 2007). In brief, clonal rat NS cell lines were generated by deposition of single cells into laminin-coated 96-well plates using a Dako Cytomation MoFlo cell sorter, followed by continuous expansion. Rat NS cells were induced to differentiate after plating  $\sim 1 \times 10^5$  cells onto a laminin-coated treated six-well plate. For astrocyte induction, cells were exposed to RHB-basal medium supplemented with N2 and 10 ng/ml of BMP4 (R&D Systems) for 7–12 days. For neuronal induction, cells were first treated for 7 days with basal medium



supplemented with B27 (1×; Invitrogen) plus 10 ng/ml FGF-2 and then switched to basal medium supplemented with N2 and B27 (0.5×) and no growth factors. For oligodendroglial induction, rat NS cells were first treated for 7 days with basal medium supplemented with B27 (1×), FGF2 (10 ng/ml) and PDGF (10 ng/ml; R&D Systems) and then switched to basal medium supplemented with N2 and B27 (0.5×) and no growth factors. For all differentiations, half the volume of medium was exchanged every 2 to 3 days.

To induce cell dormancy, we plated rat NS cells at lower density (10–30% confluence) and renewed the culture medium by 100% daily for 10–14 days. Alternatively, NS cell dormancy could be induced by applying BMP4 (10 ng/ml) in daily renewed fresh expansion medium for ~7 days. Growth curves and time-lapse videos were prepared using an IncuCyte imaging system and software (Essen Instruments).

#### Immunostaining and western blots

We used Alexa-Fluor-488- and -594-conjugated secondary conjugates (Invitrogen) and DAPI (Sigma) in immunostaining. Antibodies against the following proteins were used at the following dilutions: nestin (1:20; DSHB), Sox2 (1:400; Chemicon), GFAP (1:300; Millipore), Tuj1 (1:200; Covance), 3CB2 (1:20; DSHB), BLBP (1:500; Abcam), vimentin (1:20; DSHB),  $\beta$ -catenin (1:200; BD Biosciences), chordin (1:200; Santa Cruz Biotechnology), Ki67 (1:250; Lab Vision) and phosphorylated R-Smads (Smad1, 5 and 8) (1:200; Cell Signaling Technology). O4 staining was performed on live cells (1:100, R&D Systems).

For western blots, cell extracts (50  $\mu$ l) were resolved by SDS-PAGE (10% NuPage gel; Invitrogen) and transferred onto a polyvinylidene difluoride membrane (Amersham). The membrane was incubated with 5% non-fat dried milk powder at 4°C overnight and then probed with antibodies against the following proteins:  $\alpha$ -tubulin (1:2000; Abcam), chordin (1:200; Santa Cruz Biotechnology), Smad (1:200; Santa Cruz Biotech), phosphorylated R-Smads (Smad1, 5 and 8) (1:400, Cell Signaling), p38 (1:1000, Cell Signaling), phosphorylated p38 (1:500; Cell Signaling), PTEN (1:500; Cell Signaling), phosphorylated PTEN (1:500; Cell Signaling), Akt (1:500; Cell Signaling) and phosphorylated Akt (1:500; Cell Signaling). Membranes were incubated with a 1:5000 dilution of the appropriate horseradish-peroxidase-conjugated secondary antibody for 1 hour at room temperature. Washed blots were subjected to enhanced chemiluminescence.

#### RT-PCR and semi-quantitative real-time PCR

We used the RNeasy kit (Qiagen) to extract total RNA and Superscript III (Invitrogen) to prepare cDNA. An additional DNase step was performed on the total RNA to remove any traces of genomic DNA. cDNA concentrations were determined and normalized with NanoDrop 1000 (Thermo Scientific). RT-PCR was performed for 30 cycles for all markers except  $\beta$ -actin, which was performed for 25 cycles (denaturing for 40 seconds at 94°C; annealing for 40 seconds at 55°C, and extension for 60 seconds at 72°C). PCR products were resolved on a 1.5% agarose gel. All qRT-PCR was performed using a LightCycler (Roche). Primers were designed using MIT Primer3 software, and where possible spanned exon–intron boundaries identified with the Ensembl database. The primer sequences of candidate genes are listed in supplementary material Table S1.

#### Stable transfection of rat NS cell lines

We transfected passage 20 Ratsp2 NS cells with Scal linearized pCAGGFIP plasmid DNA (Clontech) using a Nucleofector (Amara Biosystems, program A-033) followed by puromycin selection. Viable cells with high level of GFP expression were isolated by FACS and were further expanded as Ratsp2-GFP cell line.

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Supplementary material available online at

<http://jcs.biologists.org/cgi/content/full/124/11/1867/DC1>

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